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14. ABSTRACT

The lacZ gene encoding E. coli beta-gal has already been recognized as the most commonly used reporter system in cancer gene therapy. Moreover, prostrate-specific membrane antigen (PSMA) has been identified as an ideal antigenic target in prostate cancer. We propose to develop a novel class of Gd(III)-based MRI contrast agents for in vivo detection of beta-gal or PSMA activity. This new concept of the GD(III)-based MRI contrast agents is composed of three moieties: (A) a signal enhancement group, such as Gd-DOTA or Gd-PCTA; (B) an Fe(III) chelating group; (C) beta-D-galactose or glutamate. Following cleavage by lacZ transgene or PSMA in prostate cancer cells, the released, activated aglycone Fe(III)-ligand will spontaneously trap endogenous Fe(III) at the site of enzyme activity forming a highly stable complex, to restrict motion of the GD(III) chelates enhancing relaxivity and providing local contrast accumulation. We plan to synthesize 8 novel MRI contrast agents for imaging beta-gal or PSMA activity in prostate cancer cell culture, explore the feasibility of applying the most promising analogies to cells grown in vivo in mice and rats.

15. SUBJECT TERMS
Prostate Cancer Evaluation, Contrast Agent Synthesis, MRI Gene Expression, Gene Therapy, in vivo Cancer Imaging, lacZ Gene, beta-Galactosidae, PSMA, NAALADase

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INTRODUCTION

Prostate cancer is the most commonly diagnosed cancer and the second most common cause of cancer death in men in the United States.[1,2] Gene therapy has emerged as a potentially promising strategy for treatment of prostate cancer.[3-15] The prostate is particularly amenable to gene therapy.[11-16] However, there are major issues in terms of assessing the delivery to target tissue, assessing the uniformity (versus heterogeneity) of biodistribution and determining whether the genes are expressed.[15-33] A viral construct is often readministered on successive occasions, but this should optimally be timed to coincide with loss of expression. Inevitably gene therapy has associated risks, and thus non-invasive *in vivo* determining the duration of gene expression in an individual tumor could greatly enhance the viability of the approach.

Gene expression now is commonly monitored by *in situ* hybridization techniques or by introducing a marker gene to follow the regulation of a gene of interest. Since β-galactosidase (β-gal) activity is readily assessed by histology or in culture, in hosts as evolutionarily diverse as bacteria, yeast, and mammals, its introduction has become a standard means of assaying clonal insertion, transcriptional activation, protein expression, and protein interaction, *lacZ* gene encoding *E. coli* β-gal has already been recognized as the most commonly used reporter system.[34] However, the well-established chromogenic or fluorogenic substrates, relying on the hydrolysis by β-gal to release colorful compounds are limited to histology or *in vitro* assays.[35-39] Non-invasive *in vivo* detecting of transgene expression would be of considerable value in many ongoing and future clinical gene therapy trials.

Magnetic resonance imaging (MRI) techniques recently have obtained spectacular image resolutions (voxel resolutions of about 10 μm^3 *in vitro* and about 50 μm^3 *in vivo*), opening the realm of imaging at very high resolutions in small animals during development and in clinical practice.[40-44] Additionally, a new emerging generation of responsive MRI contrast agents holds great promise in the gene therapy arena.[45,46] The abilities of these contrast agents to relax water protons is triggered or enhanced greatly by recognition of a particular biomolecule opening up the possibility of developing MRI tests specific for biomarkers indicative of particular disease states and aiding in the early detection and diagnosis of tumors. Desreux *et al* [42,47] demonstrated that, by chelating Gd(phen)HDO3A with Fe(II) to form a highly stable tris-complex, as shown in **Figure 1**, the relaxivity increased 145% at 20MHz and 37°C from 5.1$mM^{-1}s^{-1}$ per Gd(III) in Gd(phen)HDO3A form to 12.2 $mM^{-1}s^{-1}$ in the tris-complex. Desreux *et al* [42,47] also synthesized another iron-sensitive MRI contrast agent with a tris-hydroxamate (**Figure 2**). After the tris-hydroxamate groups formed a chelate with Fe(III), free rotation at the Gd(III) centers was restricted, thereby increasing relaxivity by 57% from 5.4 to 8.5$mM^{-1}s^{-1}$ at 20 MHz.

Iron is a critically important metal ion for a wide variety of cellular events.[48] Tumor cells, as compared with their normal counterparts, frequently exhibit increased uptake and utilization of iron, as evidenced by an increase in transferrin receptors at the cell surface.[49-51] Additionally, cancer cells are

sensitive to the effects of iron chelators because of the critical requirement for iron in proteins that play essential roles in DNA synthesis and energy production.[52,53] Such studies have led to iron chelation therapy to clinically treat some tumors.[54-58]

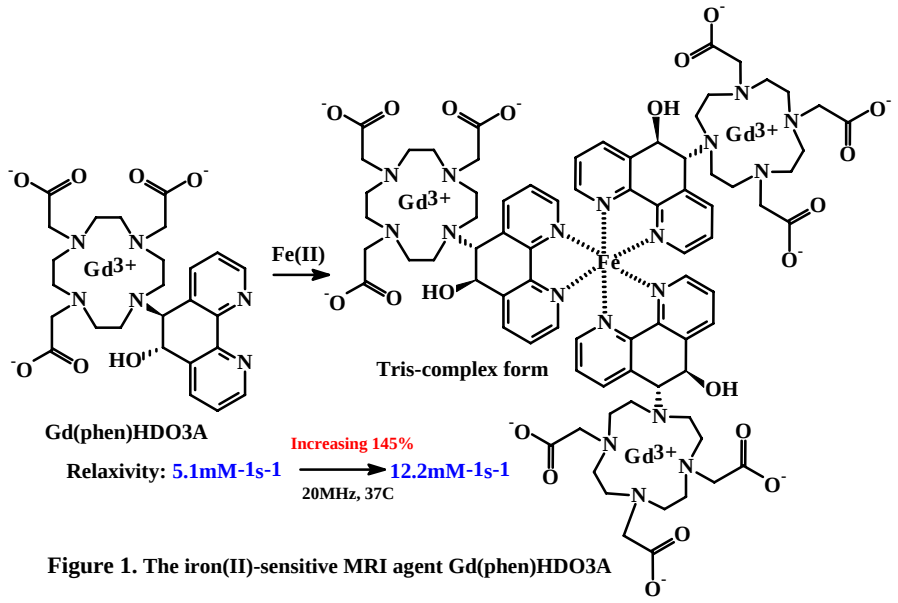


Figure 1. The iron(II)-sensitive MRI agent Gd(phen)HDO3A

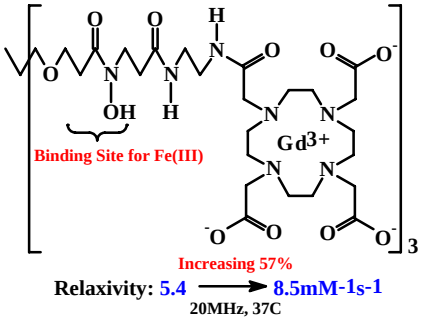


Figure 2. The iron(III)-sensitive MRI agent Gd(III)-Trishydroxamic acid

Based on the MRI contrast agents findings and the biologic features of tumor, we have proposed in this project a novel class of enzyme activated Gd^{3+}-based MRI contrast agent for *in vivo* detection of β-gal activity, in which we try to combine all means of reaching the highest possible relaxivities.[42,47] **Figure 3** depicts the mechanism for *in vivo* detection of *lacZ* gene expression through β-gal activated *in situ* Fe^{3+}-trapped MRI contrast agent formation.

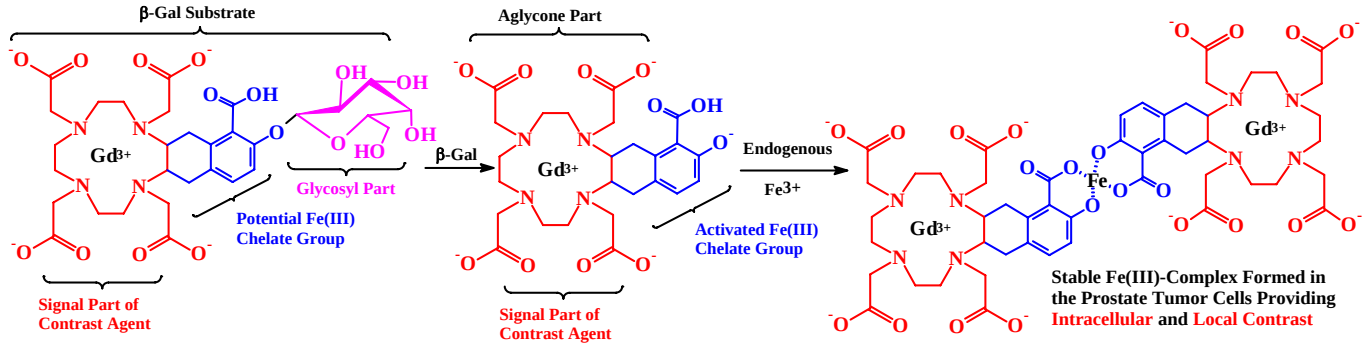


Figure 3. Mechanism of proposed new platform for *in vivo* detection of *lacZ* gene expression through β-gal activated *in situ* Fe^{3+}-trapped MRI contrast agent formation.

Additionally, prostate-specific membrane antigen (PSMA) is a type II transmembrane glycoprotein with enzymatic activities: N-acetylated α-linked L-amino dipeptidase (NAALADase) and γ-glutamyl carboxypeptidase (folate hydrolase).[59-61] Studies with the monoclonal antibodies have demonstrated that PSMA is the most well-established, highly restricted prostate cancer cell surface antigen, it is expressed at high density on the cell membrane of all prostate cancers.[62-64] The high prostate tissue specificity of PSMA has been identified as an ideal therapeutic and diagnostic target for prostate cancer,

this potential was exemplified by the recent FDA approval of an ^{111}In-labeled PSMA monoclonal antibody (Prostascint®) for diagnostic imaging of prostate cancer.[65-67] Furthermore, phase I and II trials have begun using immunotherapy directed against PSMA.[68-70] By introducing γ-glutamate residue instead of D-galactose in our proposed above new mechanism diagram, we intend to develop a novel class of Gd(III)-based MRI contrast agents for *in vivo* imaging prostate tumor through PSMA activated *in situ* Fe^{3+}-trapped MRI contrast agent formation (**Figure 4**).

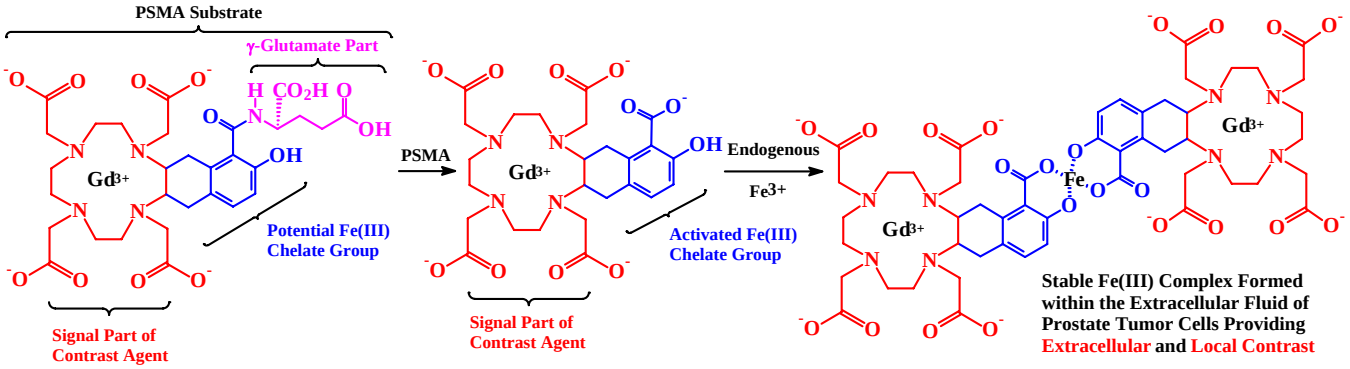


Figure 4. Proposed new mechanism for *in vivo* imaging prostate tumor through PSMA activated *in situ* Fe^{3+}-trapped MRI contrast agent formation.

Especially, PSMA has a large extracellular domain,[70] so the expression of PSMA tethered to the surface of the prostate cancer cells makes that the novel peptide-based MRI contrast agents can be targeted for activation within the extracellular fluid of prostate cancers [71] and overcomes the need for a peptide-based MRI contrast agent to penetrate the tumor cell membrane, thus, providing *in vivo* prostate cancer imaging through an **extracellular** MRI approach. The concern of permeability is one of the greatest challenges in the development of *in vivo* MRI contrast agents.[72]

Accordingly, depending upon the enzyme sources either being the *lacZ* transgene or the PSMA from prostate tumors, this new platform could provide *in vivo lacZ* gene expression assay or *in vivo* prostate cancer imaging (in particular, through **extracellular** contrast agents), with combining all the approaches of reaching the highest possible relaxivities.[42,47,72] Furthermore, this new class of responsive MRI contrast agent is composed of three functional moieties, in which the signal enhancing and Fe^{3+} chelating parts are flexible allowing modification in a search for ideal Fe^{3+}-trapped MRI contrast agents. Importantly, the combination of three functional moieties is based on the clinically applied strategies on cancer therapy. These facts strongly suggest the potential of the proposal to future clinical application.

Most recently, Merbach *et al* [73-76] also observed the remarkably high T_1 relaxivity gain by Fe(II) complex formation from (tpy-DTTA)Gd(H_2O) with 7.3$mM^{-1}s^{-1}$ to $\{Fe^{II}[Gd^{III}_2(tpy\text{-}DTTA)_2(H_2O)_4]_3\}^{4-}$ with 15.7$mM^{-1}s^{-1}$ at 20MHz and 37°C, significantly, their detailed studies on structure and dynamics of the trinuclear complex $\{Fe^{II}[Gd^{III}_2(tpy\text{-}DTTA)_2(H_2O)_4]_3\}^{4-}$ indicate that the heterometallic self-assemblies attain high T_1 relaxivities by influencing three factors: water exchange, rotation, and electron

relaxation, which are fully consistent with the expecting results shown as above in **Figures 3** and **4**, the effectiveness of contrast agents can be increased by restricting the motion of Gd(III) chelates by linking them rigidly to macromolecules through covalent or non-covalent bonds, by an improvement of their intrinsic relaxivity or by attaching several paramagnetic entities to biological or synthetic oligomers. Obviously, these comprehensive investigations as relevant evidences strongly support for our current proposal.

STATEMENT OF WORK

Specific Aim 1 Design and synthesize model "smart" MRI contrast agents to report β-gal or PSMA activities with the ability of trapping Fe^{3+} ion.

Task 1 Design and optimization of synthetic strategies for reporter molecules. (**Months 1-18**)

Task 2 Structural characterizations of the synthesized molecules. (**Months 4-20**)

Specific Aim 2 Test the properties of molecules in solution and *in vitro* with cultured prostate cancer cells.

Task 3 Evaluation the basic properties of the agents **in solution**. (**Months 20-22**)

Task 4 Evaluation of the properties of the optimal molecules *in vitro* **with cultured prostate cancer cells**. (**Months 23-25**)

Specific Aim 3 Scale up synthesis of the most promising MRI contrast agent(s) and apply to animal investigations.

Task 5 Scale up synthesis of the most promising ^{1}H MRI contrast agent(s). (**Months 26-28**)

Task 6 Apply the most promising ^{1}H MRI contrast agent(s) to assess β-gal transfection efficiency, *lacZ* gene expression (spatial and temporal) in prostate tumors *in vivo* (48 mice + 48 rats). (**Months 29-35**)

Task 7 Test dosing protocols, timing, MR detection protocols (48 mice) (**Months 29-35**)

Task 8 Prepare manuscripts and final report (**Month 36**)

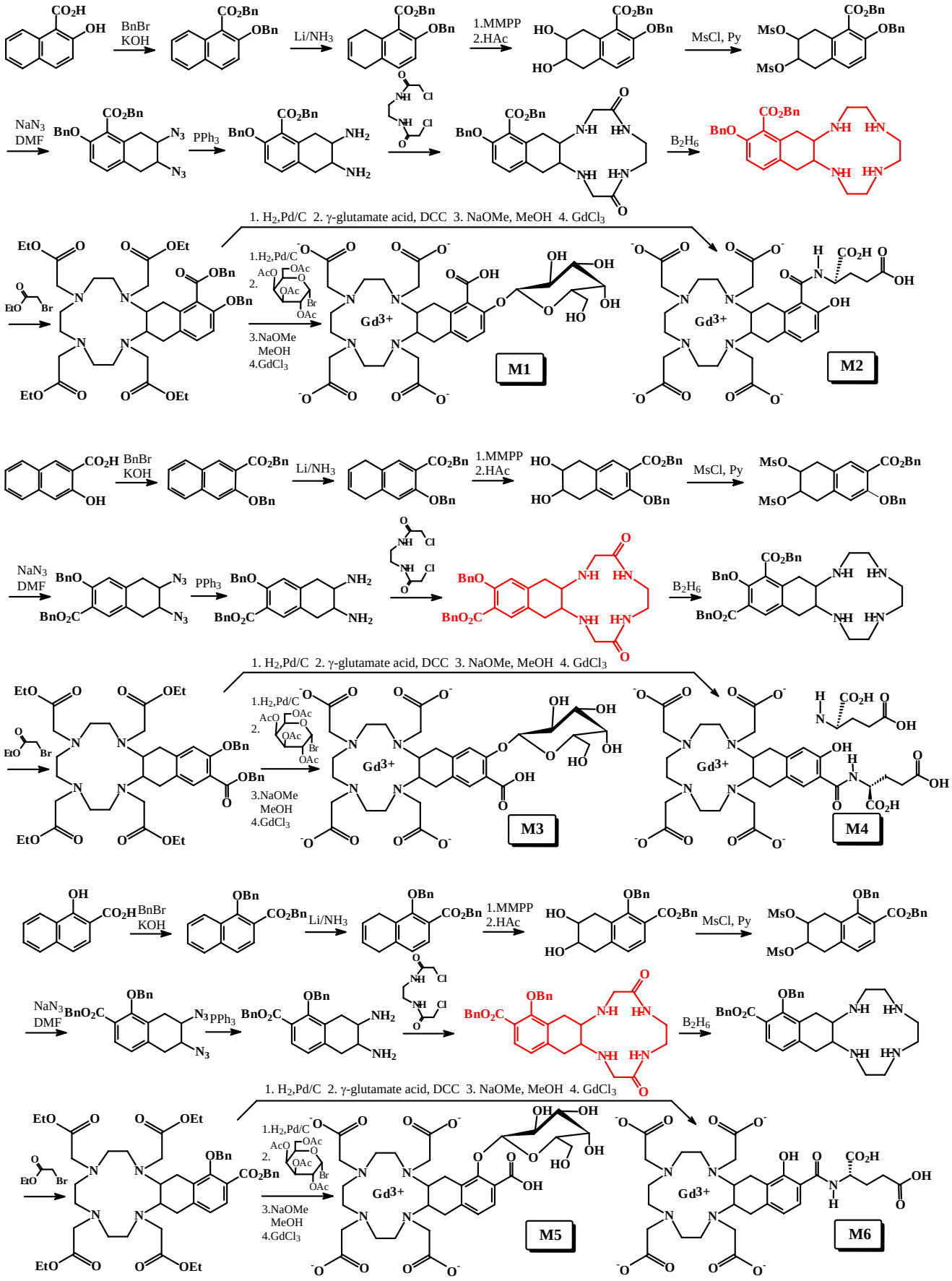
PROGRESS

In this first supported year, our work is totally focused on: **Task 1** Design and optimization of synthetic strategies for reporter molecules, and **Task 2** Structural characterizations of the synthesized molecules, strictly followed the research plan of the approved proposal W81XWH-05-1-0593.

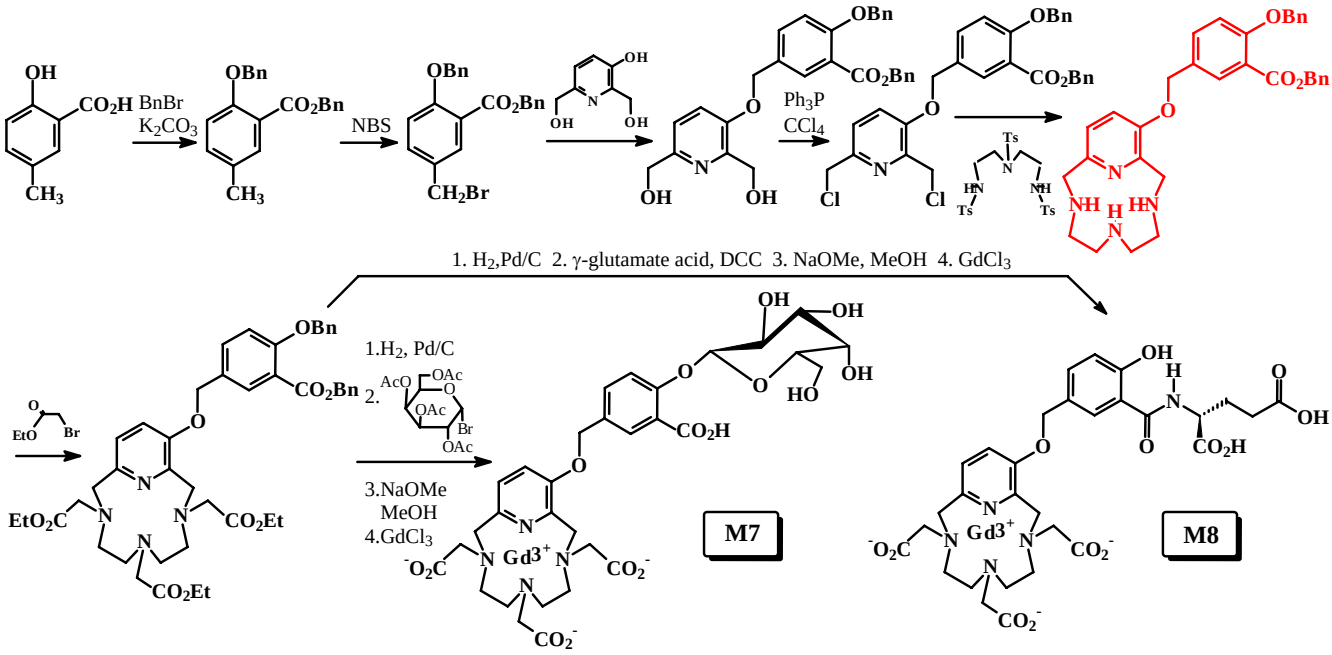
For the designed molecules $\mathbf{M_1}$ and $\mathbf{M_2}$, our syntheses have carried out according to the approaches as shown in **Figure 9** of the proposal. Through a series of reactions, we have built the key structure (*see* the red structure) of Gd^{3+} and Fe^{3+} chelators. In the next six months, we are going to stereo- and regioselectively couple with D-galactose or γ-glutamate acid.

Similarly, the syntheses of $\mathbf{M_3}$ - $\mathbf{M_6}$ have reached to the skeleton structures (*see* the red structures) of Gd^{3+} and Fe^{3+} chelators.

Prostate Cancer Evaluation: Design, Synthesis and Evaluation of Novel Enzyme-Activated ^{1}H MRI Contrast Agents



Also, the syntheses of **M_7 - M_8** by constructing 3, 6, 9, 15-tetraazabicyclo[9.3.1]pentadeca-1(15), 11, 13-triene-3, 6, 9-triacetic acid as an alternative signal enhancement group through a much different route have arrived at the key structure (*see* the red structure) of Gd^{3+} and Fe^{3+} chelators.



In each step of the multiple reactions, products were purified by chromatography or recrystallization and characterized by acquisition of ^{1}H, ^{13}C, DEPT, ^{1}H-^{1}H COSY NMR techniques.

KEY RESEARCH ACCOMPLISHMENTS

All syntheses for the target molecules **M_1 - M_8** have accomplished the construction of the important key structures of Gd^{3+} and Fe^{3+} chelators, and their structures all are verified by NMR data, providing the solid foundation for the further syntheses. Meanwhile, we have accumulated relevant experience, and gotten some expertise for efficient synthesis and separation of these intermediates, which will greatly benefit for the scale-up synthesis of the most promising ^{1}H MRI contrast agent(s) in **Task 5**.

REPORTABLE OUTCOMES

A series of intermediates related the target molecules **M_1 - M_8** have achieved.

CONCLUSIONS

Prostate cancer is the most commonly diagnosed cancer and the second most common cause of cancer death in men in the United States. The advent of effective screening measures can sharply decrease the mortality of prostate cancer through detecting this disease at an earlier stage. However, the evidence for mortality benefit from prostate cancer screening has been disappointing to date. Expanding knowledge of prostate cancer biology with combination of imaging technologies would be of considerable value in many ongoing and future clinical prostate cancer diagnosis and gene therapy trials.

Based on the biologic features of prostate cancer, we proposed in this project a new approach for *in vivo lacZ* gene expression assay or *in vivo* prostate cancer imaging (in particular, through **extracellular**

contrast agents). The ultimate objective is to demonstrate the utility and reliability of this new approach to measure β-gal or PSMA activities *in vivo*. We have accomplished the construction of the important key structures of Gd^{3+} and Fe^{3+} chelators, and verified by NMR data. We are now focusing on stereo- and regioselectively coupling with D-galactose or γ-glutamate acid to accomplish the designed molecules **M_1 - M_8,** anticipating to identify 1-2 as of the most promising MRI contrast agents for testing the sequence of tests with prostate cancer *in vitro* and *in vivo*.

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APPENDICES NONE